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LIMONENE: GREEN NON POLAR SOLVENT FOR SAMPLE PREPARATION

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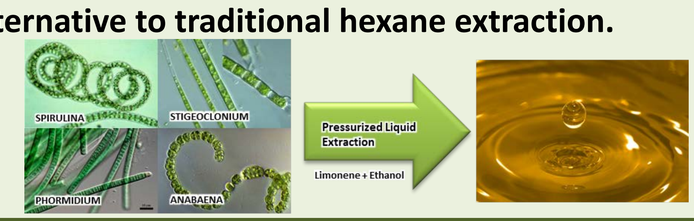
1. Introduction

- ✓ **Traditional non polar solvents like alkanes are obtained from non-renewable sources. The idea of *Green Chemistry* has its roots in sustainable development. Green Analytical Chemistry emerged from Green Chemistry in 2000 [1]. The principles of *Green Analytical Chemistry* emphasize the importance of using reagents obtained from renewable sources, eliminate toxic reagents and increase safety of the operator.**
- ✓ **The use of limonene as non-polar extracting agent in order to replace hexane has been slightly studied. Limonene is obtained from citrus peel residues, being the main compound in the terpene fraction of citrus peel oil. This compound possesses a dielectric constant close to hexane [2] and has thus been suggested as a valuable green alternative to n-alkanes and halogenated hydrocarbons**

2. Objective

To optimize a fast and green method for the isolation of high value lipids from aquatic microorganisms using Pressurized Liquid Extraction (PLE) with food grade solvents (as limonene), as an alternative to traditional hexane extraction.



3. Work flow

Compare the use of limonene vs hexane (traditional non-polar solvent) → **Optimization of extraction process with Spirulina** → **Application of the process to other microoganisms**

- **PLE:** 20.7 MPa, 180 °C, 15 min
- Limonene, limonene:ethanol
- Hexane, hexane:ethanol

- Elected solvent
- **PLE:** 20.7 MPa, 15 min
- Temperature: 50-200 ºC
- GC-MS characterization

- Cyanobacterium: Phormidium and Anabaena
- Chlorophyceae: Stigeoclonium

4. Results and discussion

❖ Limonene vs Hexane

Table 1. Changes in the extraction yield, lipid concentration and concentration of γ-linolenic acid (GLnA) in Spirulina applying pressurized liquid extraction at 20.7 MPa pressure, 180 °C temperature and 15 min extraction time using different solvents.

Solvent	Total extraction yield (%, w/w)◊	Lipids % in the extract (w/w)	GLnA % in the extract (w/w)	Lipid enrichment†	Lipid recovery†	GLnA recovery♠
Limonene	8.1 ± 0.6^{b‡}	31.4 ± 2.2^{b}	8.3 ± 0.6^{a}	3.7 ± 0.3^{a}	29.6 ± 2.1^{b}	38.4 ± 2.7^{b}
Limonene:Ethanol (1:1, v/v)	14.4 ± 1.0^{a}	34.7 ± 2.5^{ab}	7.7 ± 0.5^{a}	4.0 ± 0.3^{a}	58.1 ± 4.1^{a}	63.0 ± 4.5^{a}
Hexane	5.6 ± 0.4^{c}	35.8 ± 2.5^{ab}	8.9 ± 0.6^{a}	4.2 ± 0.3^{a}	23.3 ± 1.6^{b}	28.5 ± 2.0^{b}
Hexane:Ethanol (1:1, v/v)	13.2 ± 0.9^{a}	38.9 ± 2.8^{a}	9.0 ± 0.6^{a}	4.5 ± 0.3^{a}	60.1 ± 4.3^{a}	67.8 ± 4.8^{a}

◊Yield expressed as g of dry extract/100 g Spirulina (w/w); †♠Values relative to untreated Spirulina content of lipids (8.6%, w/w)† and GLnA (1.8%, w/w) ‡Mean ± SD (n = 2); in each column, means with different letters are significantly different (p<0.05).

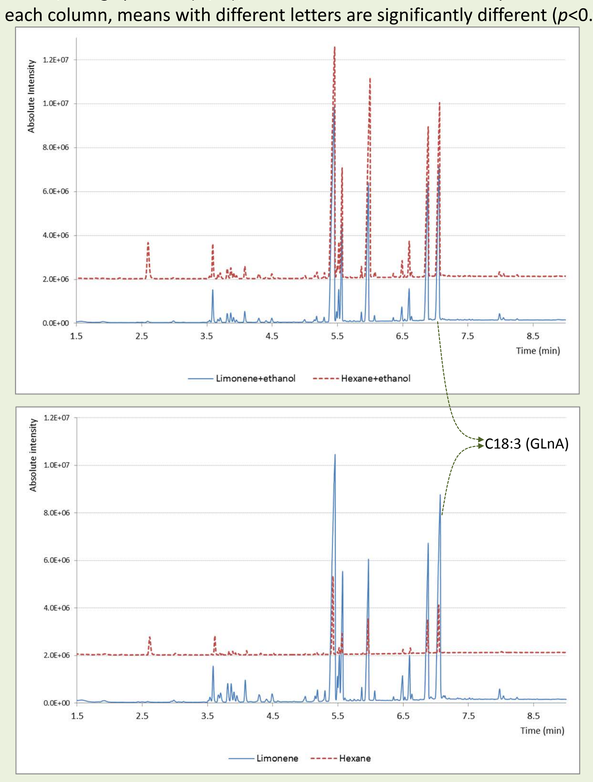


Figure 1.- Chromatograms of the fatty acid profile of Spirulina extracted with different solvents using PLE at 20.7 MPa, 180 °C and 15 min.

Elected solvent: limonene:Ethanol (1:1)

❖ Optimization of extraction process with Spirulina

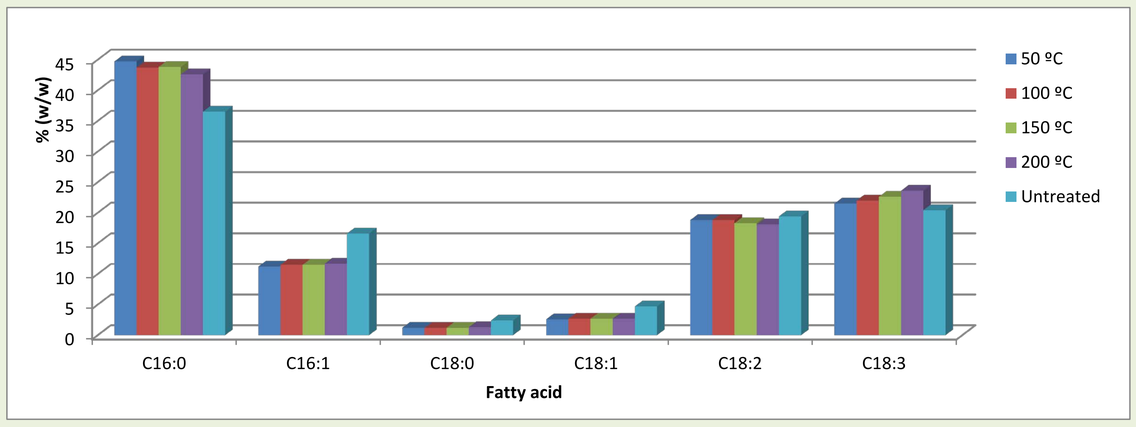


Figure 2.- Changes in the fatty acid profile of Spirulina extracted at different temperatures compared to untreated Spirulina (C16:0: Palmitic acid; C16:1: Palmitoleic acid; C18:0: Stearic acid; C18:1: Oleic acid; C18:2: Linoleic acid; C18:3: γ-Linolenic acid or GLnA).

The best conditions to obtain valuable lipidic (rich in polyunsaturated fatty acids) extracts from Spirulina within the ranges tested were: limonene:ethanol (1:1, v/v) as solvent, 200 °C of extraction temperature; 20.7 MPa as extraction pressure and a total of 15 min as extraction time.

❖ Application to other aquatic microoganisms

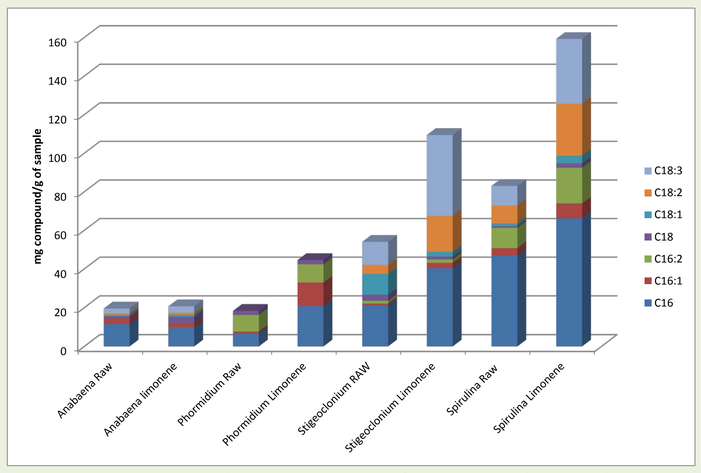


Figure 3. Fatty acid composition of extracts obtained using limonene:ethanol (1:1, v/v) as solvent, 200 °C; 20.7 Mpa and 15 min as extraction time compared to raw microorganisms.

The best results in terms of extraction of GLnA were obtained using Stigeoclonium, where this fatty acid was more than 38% w/w. For Anabaena the amount of lipids extracted was similar to the original composition of the raw sample, due to the strong cell wall [3]. Meanwhile, the extraction of lipids from Phromidium using this method was more selective since the amount of fatty acid per gram of extract was higher. This fact could be due to the high concentration of free fatty acids in Phormidium, which was previously reported [4]. In fact, Stigeoclonium could be a promising feedstock source for biodiesel production. By using the present method, the high value added lipids extracted can be employed for different purposes.

5. Conclusions

- ➢ **The proposed method (limonene:ethanol (1:1, v/v) as solvent, 200 °C of extraction temperature; 20.7 MPa as extraction pressure and a total of 15 min as extraction time) can be an interesting option to be used both for sample preparation and for lipid extract production in short time.**
- ➢ **Extracts obtained with this method are not only useful in Analytical Chemistry, they can be labeled as green extracts and directly used in food, pharmaceutical or cosmetic preparations.**

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